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Engelhardt et al.

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[54] SACCHARIDE SPECIFIC BINDING SYSTEM LABELED NUCLEOTIDES

[75] Inventors: **Dean Engelhardt; Elazar Rabbani,** both of New York; **Stanley Kline,** Brooklyn; **Jannis G. Stavrianopoulos,** New York; **Dollie Kirtikar,** Elmhurst, all of N.Y.

[73] Assignee: **Enzo Diagnostics, Inc.,** Farmingdale, N.Y.

[21] Appl. No.: **567,039**

[22] Filed: **Aug. 13, 1990**

Related U.S. Application Data

[60] Division of Ser. No. 140,980, Jan. 5, 1988, abandoned, which is a continuation of Ser. No. 674,352, Nov. 21, 1984, abandoned, which is a continuation of Ser. No. 391,440, Jun. 23, 1982, abandoned.

[51] **Int. Cl.**[5] **C07H 17/00**; C12Q 1/68

[52] **U.S. Cl.** **536/23.1**; 435/6; 536/25.32; 536/24.03

[58] **Field of Search** 435/6; 536/27

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,358,535 11/1982 Falkow et al. 435/5

FOREIGN PATENT DOCUMENTS

0063879 3/1982 European Pat. Off. .

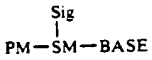
2019408 10/1979 United Kingdom .

Primary Examiner—John W. Rollins

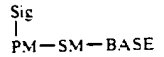
Attorney, Agent, or Firm—Ronald C. Fedus

[57] **ABSTRACT**

The present invention provides nucleotides and polynucleotides which are chemically modified or labeled so as to be capable of ready detection when attached to and/or incorporated in nucleic acid material. More particularly, this invention provides a nucleotide selected from the group consisting of (i) a ribonucleotide having the formula,



wherein PM is a phosphate moiety, SM is a sugar moiety, BASE is a pyrimidine, purine or 7-deazapurine moiety, and Sig is a saccharide moiety. PM is attached at the 2',3' or 5' position of SM. BASE is attached to the 1' position of SM from the N^1 position when BASE is a pyrimidine or covalently attached to SM; and (ii) a nucleotide having the formula.



wherein PM, SM, BASE and Sig are as defined above but PM is attached to the 3' or the 5' position of SM when said nucleotide is a deoxyribonucleotide and at the 2', 3' or 5' position when said nucleotide is a ribonucleotide. BASE is attached to the 1' position of SM from the N^1 position when BASE is a pyrimidine or the N^9 position when BASE is a purine, and wherein Sig is covalently attached to PM.

24 Claims, 7 Drawing Sheets

Fig. 2A.

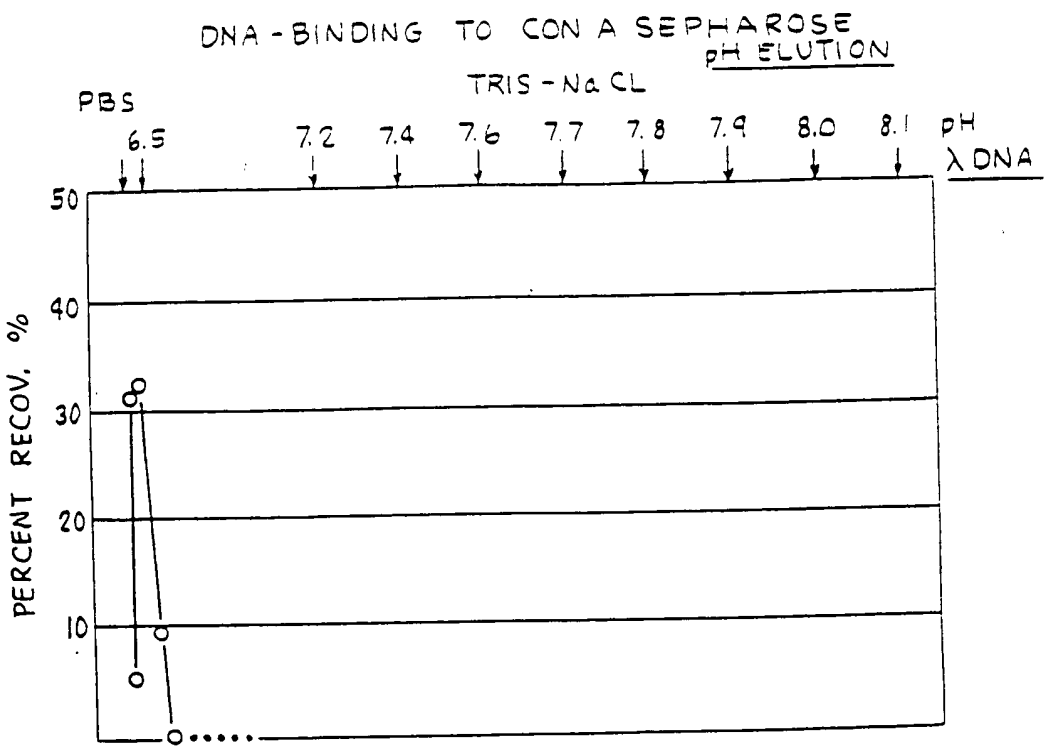

DNA - BINDING TO CON A SEPHAROSE
pH ELUTION
TRIS - Na CL
PBS
6.5
7.2
7.4
7.6
7.7
7.8
7.9
8.0
8.1
pH
λ DNA
50
40
30
20
10
PERCENT RECOV, %

FIG. 3A.

DNA BINDING TO CON A SEPHAROSE
EFFECT ON MANNOSE

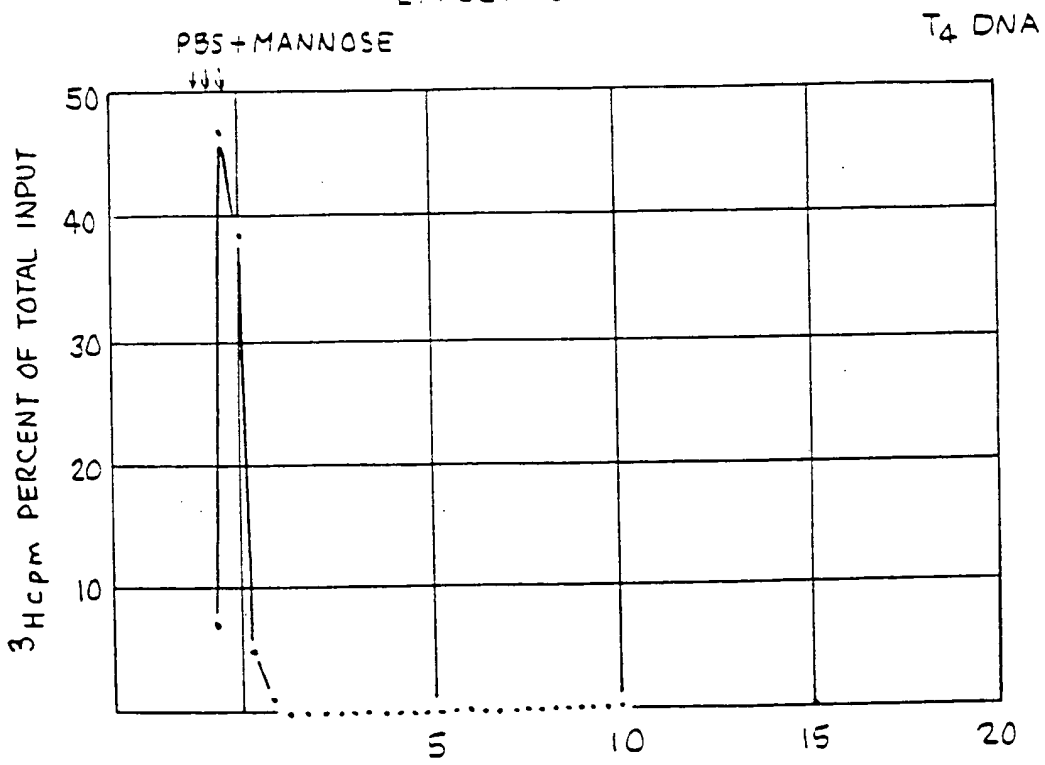
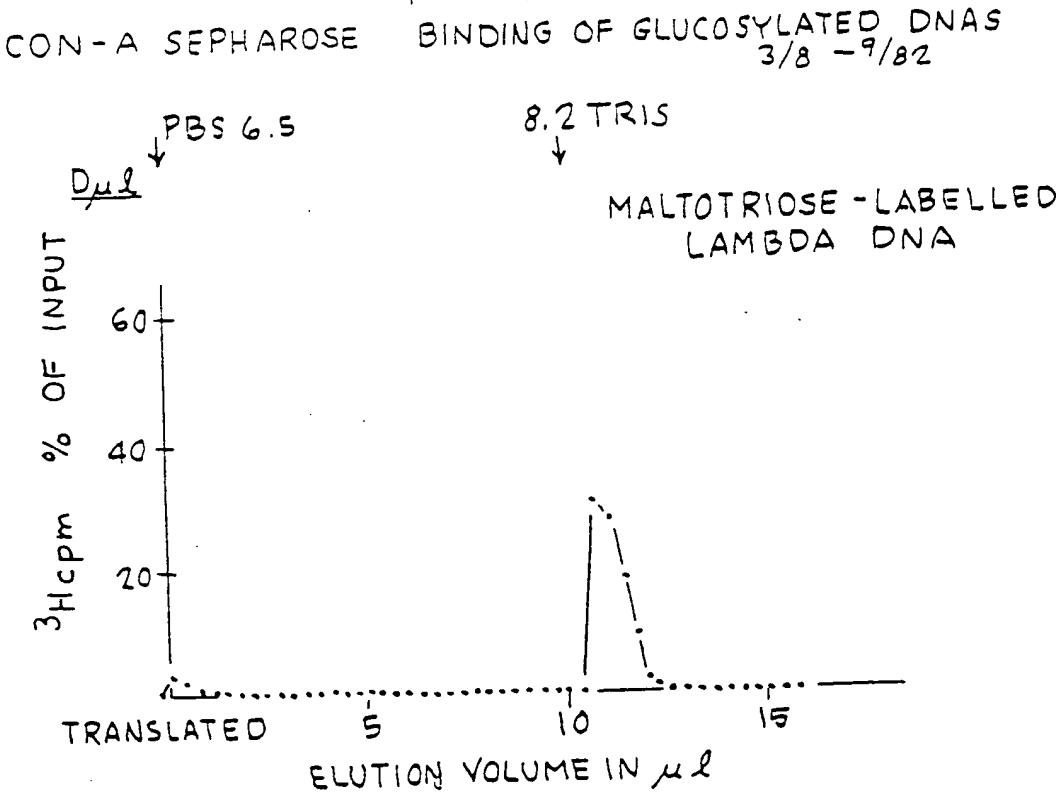


Fig. 4A.


CON-A SEPHAROSE BINDING OF GLUCOSYLATED DNAS
3/8 -9/82
PBS 6.5
8.2 TRIS
Dμl
MALTOTRIOSE-LABELLED LAMBDA DNA
% OF INPUT
60
40
20
^{3}Hcpm
TRANSLATED
5
10
15
ELUTION VOLUME IN μl

SACCHARIDE SPECIFIC BINDING SYSTEM LABELED NUCLEOTIDES

REFERENCE TO RELATED APPLICATIONS

This is a divisional application of U.S. Ser. No. 140,980, filed on Jan. 5, 1988, now abandoned, which is a continutation of US. Ser. No. 674,352, filed on Nov. 21, 1984 now abandoned, which is a continuation of U.S. Ser. No. 391,440, filed on Jun. 23, 1982 (now abandoned).

BACKGROUND OF THE INVENTION

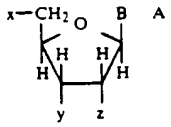
It is known to produce nucleotides or polynucleotides which are radioactively labeled, such as with isotopes or hydrogen (^{3}H), phosphorus (^{32}p), carbon (^{14}C) or iodine (^{125}I). Such radioactively labeled compounds are useful to detect, monitor, localize and isolate nucleic acids and other molecules of scientific or clinical interest. Unfortunately, however, the use of radioactively labeled materials presents hazards due to radiation. Also due to the relatively short half life of the radioactive materials employed to label such compounds or materials, the resulting labeled compounds or materials have a corresponding relatively short shelf life.

It has been proposed to chemically label compounds of interest, such as nucleotides and polynucleotides, so as to overcome or avoid the hazards and difficulties associated with such compounds or materials when radioactively labeled. In the article by P.R. Langer, A. A. Waldrop and D. C. Ward entitled "Enzymatic Synthesis of Biotin-Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes", in *Proc. Natl. Acad. Sci.*, USA, Vol. 78, No. 11, pp. 6633–6637, Nov., 1981, there are described analogs of dUTP and UTP that contain a biotin molecule bound to the C-5 position of the pyrimidine ring through an alkylamine linker arm. The biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose, even after extensive washing with 8M urea, 6M guanidine hydrochloride or 99% formamide. In addition, biotin-labeled nucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and *Staphylococcus* aurea, Protein A. These unique features of biotin-labeled polynucleotides suggest that they are useful affinity probes for the detection and isolation of specific DNA and RNA sequences. It is indicated in the article that the subject matter of the article is comprised in a pending U.S. patent application.

The disclosures of this article and above-referred pending patent application are herein incorporated and made part of this disclosure.

The patent application referred to in the above-identified article is U.S. patent application Ser. No. 255,223 filed April.17, 1981.Ser. No. 255,223 was abandoned in favor of continutation application, U.S. application Ser. No. 496,915, filed on May 23, 1983, now U.S. Pat. No. 4,711,955. Therefore, the disclosure of U.S. Pat. No. 4,711,955 is herein incorporated by reference and made part of the instant disclosure. The disclosures of this pending U.S. patent application Ser. No. 255,223 are herein incorporated and made part of this disclosure. In the above-identified pending U.S. patent application the subject matter of the above-identified article is disclosed and additionally it is disclosed that

compounds having the structure:

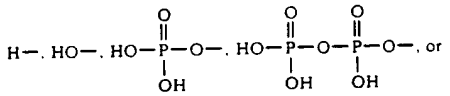


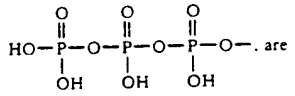
wherein B represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the C^{1}-position of the sugar moiety; provided that when B is purine or 7-deazapurine, it is attached at the N^{9}-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^{1}-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y, and z represents





widely useful as probes in biomedical research and recombinant DNA technology.

Particularly useful are compounds encompassed this structure which additionally have one or more of the following characteristics: A is non-aromatic; A is at least C_5; the chemical linkage joining B and A includes an α-olefinic bond; A is biotin or iminobiotin; and B is a pyrimidine or 7-deazapurine.

The publications cited in the aforementioned U.S. Pat. No. 4,711,955 are also herein incorporated and made part of this disclosure.

SUMMARY OF THE INVENTION

In accordance with the practices of this invention nucleotides are modified, such as at the 5 position of pyrimidine or the 7 position of purine, preparatory for the preparation therefrom of nucleotide probes suitable for attachment to or incorporation into DNA or other nucleic acid material. In the practices of this inven tion nucleotides, i.e. nucleic acids, preferably are modified in a non-disruptive manner such that the resulting modified nucleotides are capable of incorporation into nucleic acids and once incorporated in nucleic acids the modified nucleotides do not significantly interfere with

EXAMPLE IV

Oligodeoxyribonuclectides were end-labeled using cytidine-5'-triphosphate and terminal transferase as follows. Purified phage DNA, alkali sheared with 0.2 N sodium hydroxide and diluted to 2 A_{260} units/ml in potassium cacodylate (0.1 M), tris base (25 mm), cobalt chloride (1 mM) and dithiothreitol (0.2 M) were used. To this DNA solution (1 ml) was added cytidine-5'-triphosphate (10 mmol) and terminal transferase (200 units). After incubating at 37° for 5 to 8 hours the reaction was stopped by the addition of neutralized phenol (100 ul), 0.5 M EDTA (100 ul) and 1% sodium dodecyl sulfate (100 ul). The DNA was purified by gel filtration chromatography through Sephadex G-100 followed by precipitation with ethanol.

EXAMPLE V

Biotin and polybiotinylated poly-L-lysine were coupled to oligoribonucleotides using a carbodimide coupling procedure described by Halloran and Parker, *J. Immunol.*, 96 373 (1966). As an example, DNA (1 ug/ml), 1 ml) in tris buffer pH 8.2, sheared with 0.1 N sodium hydroxide was denatured by boiling for 10 minutes and quick cooling in an ice bath. Biotinyl-1,6-diaminohexane amide (2 mg, 6 umol) or polybiotinylated poly-L-lysine (2 mg) and 1-ethyl-3-diisopropylaminocarboimide HCl (10 mg, 64 umol) were added, and the pH readjusted to 8.2. After 24 hours at room temperature in the dark, the mixture was dialyzed against 10 mM tris buffered saline. DNA was precipitated ethanol.

EXAMPLE VI

Biotin, conjugated to cytochrome C, was prepared by tile following procedure. To a solution of cytochrome C (10 mg) in 1 ml of 0.1 M sodium borate, pH 8.5 was added biotinyl-N-hydroxysuccinimide ester (10 mq, 29 umol) in 1 ml dimethyl formamide. After 4 hours at room temperature, the biotinylated protein was purified by gel filtration chromatography through a Sephadex G-50 column.

EXAMPLE VII

Formaldehyde coupling of cytochrome C-biotin and polybiotinylated poly-L-lysine to oligodeoxyribonucleotides were carried out using a method similar to that described by Manning et al. *Chromosoma*, 53, 107 (1975). Oligodeoxyribonucleotide fragments obtained by sodium hydroxide shearing of purified DNA (100 ug/ml in 10 mM triethanolamine, pH 7.8 were denatured by boiling for 10 minutes followed by quick cooling in ice. Cytochrome C-biotin 0.05 g ml or polybiotinylated poly-L-lysine solution (0.05 ml) dissolved 3 mg/ml in 10 mM triethanolamine, pH 7.8 was added to 1 ml at the denatured oligodeoxyribonucleotide solution along with 0.1 ml of 6% formaldehyde in 10 mM triethanolamine, pH 7.8. After stirring at 40° for 30 minutes the mixture was dialyzed against the same buffer. The oligodeoxyribonucleotidebiotin complex was finally purified by gel filtration chromatography on Sephadex G-100 followed by precipitation from ethanol.

EXAMPLE VIII

Double stranded polydeoxyadenylic acid:polybiotinylated deoxyuridylic acid was synthesized as follows. The double stranded oligonucleotide polydeoxyadenylic acid:polythymidylic acid (20 ug) of length 300 basic pairs, dissolved in 200 ul exonuclease III buffer consisting of Tris-HCl pH 8.0 (70 mM); magnesium chloride (1.0 mM) and dithiothreitol (10 mM) was incubated with 100 units exonuclease III for 20 minutes at 20° C. The partially digested oligonucleotide was immediately extracted with phenol, and the DNA was precipitated with 70% aqueous ethanol. The partially digested oligonucleotide was redissolved in 20 ul 5mM tris-HCl pH 7.6 and incubated at 20° C. for 2 hours in a reaction containing 2'--deoxy- adenosine-5'-triphosphate (15 uM) thymidine-5'-triphosphate (the amount determines the degree of substitution) and biotinylated 5-(3-amino-1-propene) 2'-deoxyuridine-5'-triphosphate (5 uM), Klenow DNA polymerase I (200 units) dissolved in 0.1 mM) potassium phosphate, pH 8.0 at a concentration of 0.2 units/ul. The biotinylated poly dA:poly dT, biotinyl dU was purified by gel filtration chromatography on Sephadex G-100. The DNA was enthanol precipitated and redissolved in 20 ul of solution containing sodium acetate pH 4.6 (30 mM), sodium chloride (50 mM), zinc sulfate (1mM) and glycerol (5%). S_1 nuclease (200 units) was added, and the reaction was incubated at 37° for 10 minutes. The reaction was stopped with 1 ml ammonium acetate (4 M) and 6 ml ethanol. The DNA was repurified by G-100 gel filtration chromatography and ethanol precipitation.

EXAMPLE IX

Ligation of poly dA:poly dT, biotinyl dU to oligodeoxyribonucleotides was accomplished as follows: DNA fragments from alkali sheared purified DNA (as described in Example VIII) were digested with S_1 nuclease and repurified by phenol extraction and ethanol precipitation. Blunt ended DNA fragments (1 ug) and poly dA:poly dT, biotinyl dU (2 ug) were dissolved in 6 ul at a buffer containing tris-HCl pH 7.4 (66 mM), magnesium chloride (6.6 mM), adenosine triphosphate (24 mM) and dithiothreitol (l.OmM), T_4 DNA ligase (50 units) was added, and the volume brought to 20 ul with water. The reaction was incubated 3 hours at 37° C. The DNA was purified by gel filtration chromatography through Sephadex G-100 and was ethanol precipitated.

EXAMPLE X

5-Hydroxymethyl-2'-deoxycytidylic acid was prepared by enzymatic hydrolysis of non glycosylated phage T_4 DNA. Purified phage DNA (2 mg), dissolved in 1 ml 50 mM tris pH 7.4 and 10 mM magnesium chloride, was incubated 20 hours with deoxyribonuclease I at 37°. The pH was adjusted to 9.0 and sodium chloride (20 mM) added. Snake venom phosphodiesterase (0.05 g units in 0.5 ml water) was added and incubation continued at 37° for 5 hours. An additional 0.05 units phosphodiesterase was added and incubation continued 18 hours. Nucleotides were separated by gel filtration chromatography through Sephadex G-50. 5-hydroxymethyl-2'-deoxycytidylic acid was purified by reverse phase high pressure liquid chromatography.

EXAMPLE XI

5-(4-aminobutylaminomethyl)-2'-deoxyuridylic acid was obtained by enzymatic hydrolysis of DNA from phage OW-14. The phage was grown on *Pseudomonas acidovorans* 29 according to Kropinski and Warren, *Gen. Virol.* 6, 85 (1970),and the phage DNA purified according to Kropinski et al. *Biochem.* 12, 151 (1973). The DNA was enzymatically hydrolyzed with deoxyribonuclease I and snake venom phosphodiesterase using

EXAMPLE XIX

5-substituted pyrimidine triphosphates were chemically prepared from their respective 5' monophosphates using a procedure of Michelson, *Biochem Biophys* Acta, 91, 1, (1964). The example of 5-hydroxymethyl-2'-deoxycytidine-5'-triphosphate will be given. The others were similarly prepared. 5-hydroxymethyl-2' deoxycytidylic acid (free acid) (0.63 g, 0.2 mmol) was converted to its tri-n-octylammonium salt by suspending in methanol and addition of tri-n- octylammonium hydroxide (0.74 g, 0.2 mmol). The suspension was refluxed until a clear solution was obtained and the solvent removed under vacuum. The salt was dried by dissolution in and subsequent evaporation from dry pyridine several times. To the salt, dissolved in dry dimethylformamide (0.1 ml) and dioxane (1 ml) was added diphenylphosphochloridate (0.1 ml) and tri-n-butylamine (0.2 ml); After 25 hours at room temperature, solvent was removed and ether was added to precipitate the nucleoside-5'-diphenylpyrophosphate. This was dissolved in dioxane (0.5 ml) and a solution of di(tri-n-butylammonium) pyrophosphate (0.5 mmol) in 1 ml pyridine was added. After 45 minutes at room temperature, the mixture was conentrated under vacuum to a small volume. The crude product was precipitated with ether. This was dissolved in 0.1 M phosphate buffer pH 8.0. The trisphosphate was purified by chromatography on DEAE cellulose eluting with a gradient of 0.1 to 0.6 M triethylammonium bicarbonate ph 7.5.

EXAMPLE XX

DNA was labeled with 5-substituted pyrimidine triphosphates by nick translating DNA in the presence of the appropriate triphosphate. An example follows for labeling purified DNA with biotinylated 5-formyl-2'-deoxyuridine. DNA (20 ug/ml) was incubated at 14° C. in the presence of magnesium chloride (5 mM) 2'-deoxycytidine-5'-triphosphate (15 mM), 2+deoxyadenosine-5'-triphosphate (15 uM), 2'-deoxyguanosine-5'-triphosphate (15 uM), biotinylated-5-formyl-2'-deoxyuridine-5'-triphosphase (20 uM), activated pancreatic deoxyribonuclease I (13 mg/ml), *E. coli* deoxyribonuclease acid, polymerase I (40 units/ml) and tris HCL, PH 7.4 (50 mM). Alter 2 hours the reaction was stopped by addition of 0.3 M EDTA (0.05 ml) followed by heating at 65° for 5 minutes. Labeled oligonucleotide was purified by gel filtration chromatography through Sephadex G-100 and precipitation from cold ethanol.

EXAMPLE XXI

PRECIPITATION OF GLUCOSYLATED DNA BY CONCANAVALIN A

Reaction mixtures (1.0 ml) were prepared in 1.5 ml eppendorf tubes as follows:

Sodium potassium phosphate, pH 6.5	10 mM
NaCl	150 mM
$MgSO_4$	5 mM
$CaCl_2$	1 mM
DNA (T4 of calf thymus)	50 ug
Cancanavalin A (10 mg. ml)	50–500 ug

Reactions were started by the addition of concanavalin A (Con A). The solutions were mixed and left at room temperature for 60 minutes. The tubes were centrifuged at 1200 g for 15–20 minutes. The supernatants were diluted and the A260 was measured.

Since Con A absorbs at 260 nanometers, control solutions lacking DNA but containing Con A were prepared. The Con A absorbance was substracted from the absorbence obtained in the complete reaction mixtures.

The results of this reaction are presented in accompanying FIG. 1.

EXAMPLE XXII

BINDING OF GLUCOSYLATED DNA TO CONCANAVALIN A

Phage T4 DNA and phage DNA were labeled by incorporation of H^3-deoxyadenosine triphosphate into the DNA by nick translation according to the Rigby et al procedure. T4 DNA was nick translated to a specific activity of 5×10^5cpm/microgram and an average double- standed size of 5 kilobases. Lambda DNA was nick translation to a specific activity of 3×10^5cpm/microgram and an average. double stranded size of 6.0 kilobases as determined by agarose gel electrophoresis. Unincorporated nucleotides were removed from the reaction mixtures by Bio-Gel P-60 chromatography.

Con A sepharose was prepared as described by the manufacturer (Pharmacia). One ml of settled gel contained 18 mg of bound Con A. One ml columns were prepared in sterile pasteur pippetes and were equilibrated with PBS (0.15 M NaCl; 0.01 M sodium potassium phosphate, pH 6.5).

H^3-DNA samples were prepared in 0.5 ml of buffer (as described in Example XXI but without Con A). T4 DNA solutions contained 176.000 cpm/0.5 ml; and DNA solutions contained 108,000 cp./0.5 ml. A 0.5 ml sample was applied to the column.

A 10.5 ml volume of buffer was passed through the column, and the eluate fractions (0.33 m) were collected and counted in a Beckman LSC-100 scintillation counter in a 3.5 ml reafluor cocktail (Beckman). The results (FIGS. 2A and 2B) show that non-glucosylated DNA was not bound whereas glucosylated T4 DNA was bound to the column. The bound T4 DNA was removed by washing the column with a higher pH buffer (Tris-HCl, PH 7.2–8.2).

Furthermore, consistent with the interaction of glucose and Con A, mannose, when included in the buffer in which the DNA is applied to the column, prevents binding of glucosylated DNA to Con A sepharose. Also, mannose-containing buffer (PBS-containing 0.056 M mannose) removes bound T4 DNA from Con A sepharose (FIGS. 3A and 3B).

Further illustrative of the practices of this invention directed to nonradioactive methods or techniques of assaying for specific nucleic acids, the following example deals with the use of the sugar-lectin system. This example deals with the use of DNA which is not glycosylated in nature but rather has had a maltotriose group added thereto by way of nick translation described herein. The maltotriose modified dUTP and DNA modified therewith bind specifically to a column of concanvalin A covalently bound to sepharose. By this technique and in accordance with the practices of this invention, there is provided a means for specifically labeling any nucleic acid with sugars. As previously indicated herein, nick translation is only one of a number of techniques and approaches possible for the production of the modified nucleic acids in accordance with this invention.

incubated at 40° C. for 1 hour and at room temperature for 18 hours. It was then loaded on a DEAE-cellulose column and eluted with a gradient of 0.1 to 0.6M triethylammonium bicarbonate, pH 7.5. The product was finally purified by reverse phase high pressure liquid chromatography.

Following are Examples XXXII and XXXIII. Example XXXII is a method of tagging allylamine modified DUTP with a fluorescein substituent. Thus is an example of creation of a self detecting nucleic acid probe. Example XXXIII is a method of labeling preformed double helical nucleic acids at the N^2 position of guanine and the N^6 position of adenine. Example XXXVII has the detector molecule linked to the probe. Chromosoma 84: 1-18 (1982) and Exp. Cell Res. 128:485-490, disclose end labeling of RNA with rhodamine. However, the procedure of this invention is less disruptive and labels internal nucleotides.

EXAMPLE XXXII

Fluorescein was coupled to 5-(3-amino-1-propyl)-2'-deoxyuridine-5'-triphosphate (AA-dUTP) as follows. AA-DUTP (10 umol), dissolved in 2 ml sodium borate buffer (0.1 m), pH 9.0, was added to fluorescein isothiocyanate (10 mg, 25 umol) dissolved in 1 ml dimethylformamide. After four hours at room temperature the mixture was loaded onto a DEAE-cellulose column equilibrated in triethylammonium bicarbonate buffer, pH 7.5. The fluorescein coupled AA-DUTP was purified by elution with a gradient of from 0.1 to 0.6 m triethylammonium bicarbonate, pH 7.5.

EXAMPLE XXXIII

DNA may be modified by reaction with chemical alkylating agents. Lambda DNA was alkylated in N^2 position of guanine and N^6 position of adenine by reacting DNA with aromatic hydrocarbon 7-bromomethylbenz[a]anthracene. 7-bromomethylbenz[a]anthracene was obtained as follows. 7-methyl[a]anthracene in carbon disulfide solution was cooled in a freezing mixture and treated dropwise with a molar equivalent of bromine. After 30 minutes, the product in suspension was collected, and was washed with dry ether and recrystallized from benzene. The yield was 66% with melting point 190.5°-191.50° C.

DNA, purified from phage Lambda, (1.6 mg) was solubilized in 5.0 ml of 20 mM potassium phosphate pH 6.5. To 4.0 ml of DNA solution was added 500 micrograms 7-bromomethylbenz[a]anthracene in dry acetone. After 30 minutes at 20°, the DNA was precipitated with two volumes of cold ethanol. The precipitate was washed successively with ethanol, acetone and ether to remove any unbound 7-bromomethylbenz[a]anthracene. Enzymatic hydrolysis of the DNA to nucleosides and subsequent chromatography of the products on Sephadex LH-20 columns, indicated that 18% of the adenine and 48% of the guanine in DNA were modified in N^6 and N^2 positions, respectively.

The modified DNA was made single stranded either by (1) heating to 100° for 5 minutes and rapid cooling or (2) incubating with equal volume of 0.1M NAOH for 10 minutes and then dialyzing the solution for four hours against 1 ml tris-HCl pH 8.0 containing 0.5 ml EDTA to keep the DNA in single-stranded form.

EXAMPLE XXXIV

A DNA probe was ligated to a synthetic DNA composed of repeated sequences of *E. coli* lac operator DNA. After hybridization to detect antiprobe sequences, the hybridized DNA was detected by reaction with biotinylated lac repressor which was, in turn, detected by an enzyme linked immuno sorbent assay using goat antibiotin IGG to react with the biotin and a second antibody coupled to horse radish peroxidase. The lac polyoperator DNA has been described by Caruthers (Second Annual Congress for Recombinant DNA Research, Los Angeles, 1982), and it was ligated, in a blunt end ligation, using T4 ligase, to an adenovirus DNA probe. In situ hybridization of the polyoperator-labeled probe DNA was carried out as described by Gerhard et al (*Proc. Natl. Acad. Sci.* USA, 78, 3755 (1981). Biotinylated lac repressor was prepared as described by Manning et al (*Chromosoma*, 53, 107-117 (1075) and was applied to adenovirus infected cells, fixed to a glass slide, in Binding buffer composed of (0.0 MK Cl, 0.01M tris (pH 7.6), 0.01M $MgSO_4$, 10^{-4} MEDTA, 10^{-4}M DTT, 5% DMSO (dimethyl sulfoxide) and 50 μg/ml bovine serum albumin by J. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory (1972). The slides were washed in binding buffer to remove unbound biotinylated lac repressor and then assayed for biotin using the horse radish peroxidase-linked double antibody procedure. This procedure could be adapted to create an affinity column where the probe could be bound to immobilized repressor protein and then removed by elution with a specific inducer, for example, isopropylthigalactoside or thiomethylgalactoside. The affinity of the repressor- operator complex is quite high 10^{-11}M. When a specific inducer binds to the repressor the operator-repressor complex collapses.

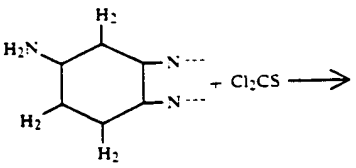
EXAMPLE XXXV

5-Bromo-2'-deoxyuridine-5'-phosphate was prepared as follows: 2'-Deoxyuridine-5'-phosphate (6.2 g) was suspended in a mixture of 60 ml pyridine and 30 ml acetic acid. Bromine (0.84 ml) was added with stirring in an ice water bath and stirring continued for 20 hours at room temperature. The solution was concentrated by vacuum. After redissolution in a minimum of water a crude product was precipitated by addition of ethanol. The crude product was chromatographed on Dowex 50 (H^+) and eluted with water. The free acid product was precipitated from the concentrated eluent by addition of ethanol.

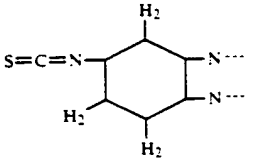
EXAMPLE XXXVI

Calf intestine alkaline phosphate was biotinylated as follows: The enzyme (1 mg, 7.7 mmol) was chromatographed on a G-50 column eluting with 0.1M Hepes buffer pH 8.0 containing 0.1M sodium chloride. The pooled fractions were reacted with N-biotinyl-6-aminocaproic acid-N-hydroxysuccinimide ester (0.675 mg, 0.77 umol) dissolved in 10 ml diemthylformamide at room temperature for 1 hour. Sodium periodate (0.1M 125 μl) was added and stirring continued for 2 hours. The mixture as dialyzed at 4° overnight in 0.1M Hepes buffer pH 8.0 with 0.1M NaCl after which the PH was adjusted to 7.4. Biotin hydrazide (0.1M, 0.5 ml) dissolved in 0.1M Hepes buffer pH 7.4 and 0.1M NaCl was added and the reaction stirred for 30 minutes at room temperature. The pH was adjusted to 8.0 with 0.2M sodium carbonate and 0.5 ml of freshly prepared 0.1M sodium borohydride in water was added, the solution was dialyzed against 0.1M tris buffer pH 8.0 with 0.1M NaCl.

Then this compound is reacted with chloro thiophosgene to produce the isothiocyanate derivative of (TCM-dANCH).

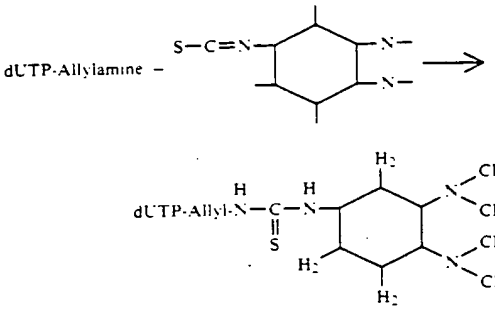


5)



Finally, this compound is reacted with dUTP-allylamine dprivative to produce modified dUTP.

6)



Cobalt or other heavy metal ions or other rare earth ions can be chelated to the compound after step 3 above. Or the nucleic acid can be substituted with this adduct and then the ion added. (Example. cobalt is added at pH 6 where the binding constant is 10^{-19}M).

Cobalt can be assayed by radioactivity. It can also be detected by its ability to oxidize methylene blue to the leuco form in the presence of molecular oxygen. It can be used to oxidze soluble sulfhydro groups to disulfide bonds again in the presence of molecular oxygen.

This type of self-signaling molecule can be used to monitor any nucleic acid hybridization reaction. It is particularly important for detecting nucleic acids in gels (for example. sequencing gels).

With respect to its use in radioactivity, it can be used to tailor the isotope needed,, i.e. if a weak or strong β or γ emitter is needed. that isotope can be chelated. Examples of isotopes that can be used are listed immedlately hereinafter.

Antimony-124	Iodine-125	Scandium-44
Antimony-125	Iodine-131	Scandium-46
Arsenic-74	Iodine-132	Selenium-75
	Indium-192	Silver-110 m
Barium-133	Iron-55	Silver-111
Barium-140	Iron-59	Sodium-22
Beryllium-7		Strontium-85
Bismuth-206	Krypton-85	Strontium-89
Bismuth-207		Strontium-90
	Lead-210	Sulphur-35
Cadmium-109	Lutecium-177	
Cadmium-115 m		Tantalum-182
Calcium-45	Manganese-54	Technetium-99

-continued

Carbon-14	Mercury-197	Tellurium-125 m
Cerium-139	Mercury-203	Tellurium-132
Cerium-141	Molybdenum-99	Terbium-160
Cerium-144		Thallium-204
Cesium-134	Neodymium-147	Thorium-228
Cesium-137	Neptunium-237	Thorium-232
Chlorine-36	Nickel-63	Thulium-170
Chromium-51	Niobium-95	Tin-113
Cobalt-56		Titanium-44
Cobalt-57	Osmium-185 + 191	Tritium
Cobalt-58		Tungsten-185
Cobalt-60	Palladium-103	
		Vanadium-48
Erbium-169	Platinum-195 m	Vanadium-49
Europium-152	Praseodymium-143	
	Promethium-147	Ytterbium-169
Gadolinium-153	Protactinium-233	Yttrium-88
Gold-195		Yttrium-90
Gold-199	Radium-226	Yttrium-91
	Rhenium-186	
Hafnium-175	Rubidium-86	Zink-65
Hafnium-175 + 181	Ruthenium-103	Zirconium-95
Hafnium-181	Ruthenium-106	
Hydrogen-3 see Tritium		

Streptavidin. a protein produced by a *Streptomyces avidinii* is a large molecular weight component of a synergistic pair of compounds which are both present in the culture filtrates of this microorganism. Each of the pair is inactive but in combination are active against gram-negative microorganisms. It has been found that the small component of this antibiotic prevents de novo synthesis of the vitamin biotin and thus. at least in synthetic media. show antimicrobial activity. In complex medium. however, the large component has to be included to exert the same effect on bacteria. This has been shown to be due to the presence of external biotin in the complex medium. The large molecular component has been found to bind external biotin and thus demonstrating the same kind of action as avidin from eggs and oviduct tissues of laying birds.

Streptavidin has been purified and shown to be a 60,000 dalton polypeptide. Like avidin. streptavidin contains four subunits and binds tightly four molecules of biotin. Unlike avidin. however, it is non-glycosylated and it has PI of 5.0 as compared Lo avidin with PI = 10.5. Due to the difference in pI streptavidin does not have a tendency to non-specifically interact with DNA.

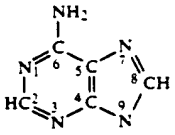
PREPARATION OF STREPTAVIDIN

A semi-synthetic medium containing salt, 1% qlucose, 0.1% asparagine, 0.05% yeast extract and trace elements was prepared. The cultures were grown at 26° C. for three days. Mycellium was removed by centrifugation and protein in the supernatant were absorbed to DEAE-cellulose in a batchwise process after pH was adjusted with 1M HCl to 7.2. DEAE-cellulose was filtered off and washed with 20 mM Tris-HCl (pH 7.2) until no absorbancy at 280 nm was recorded. Streptavidin was eluted with 20 mM Tris-HCl (pH 7.2) containing 0.5M NaCl. Ammonium sulfate precipitation was used to further concentrate the streptavidin (50% w/v at 4° C.).

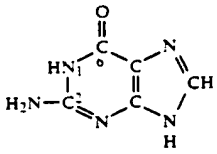
The precipitate was dissolved in water and dialyzed against 1.0M NaCl. 50 mM Na_2CO_3. In the next step affinity column chromatography on iminobiotin sepharose was used. Eluted streptavidin Erom iminobiotin sepharose column was shown to be chromatographi-

-continued

Adenine
(6-aminopurine)

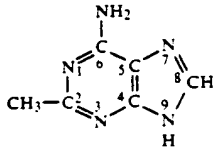


Guanine
(2-amino-6-oxypurine)

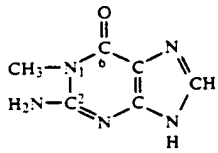


Two minor purines

2-Methyladenine

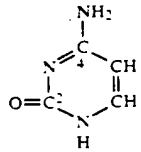


1-Methylguanine

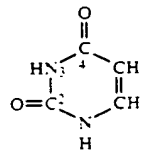


The major pyrimidines

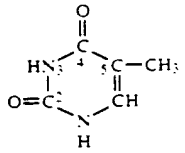
Cytosine
(2-oxy-4-aminopyrimidine)



Uracil
(2,4-dioxypyrimidine)

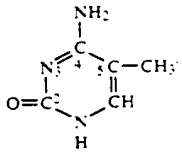


Thymine
(5-methyl-2,4-dioxypyrimidine)

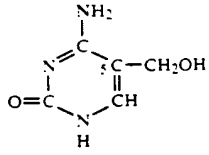


Two minor pyrimidines

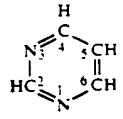
5-Methylcytosine



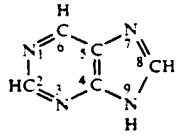
5-Hydroxymethylcytosine



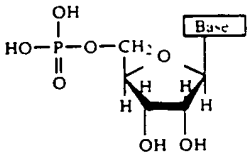
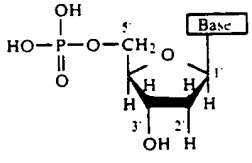
PYRIMIDINE



PURINE



The major ribonucleotides and deoxyribonucleotides.

Ribonucleoside 5'-monophosphates	2'-Deoxyribonucleoside 5'-monophosphates
	

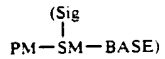
General Structure Names	General Structure Names
Adenosine 5'-phosphoric acid (adenylic acid; AMP)	Deoxyadenosine 5'-phosphoric acid (deoxyadenylic acid; dAMP)
Guanosine 5'-phosphoric acid (guanylic acid; GMP)	Deoxyguanosine 5'-phosphoric acid (deoxyguanylic acid; dGMP)
Cytidine 5'-phosphoric acid (cytidylic acid; CMP)	Deoxycytidine 5'-phosphoric acid (deoxycytidylic acid; dCMP)
Uridine 5'-phosphoric acid (uridylic acid; UMP)	Deoxythymidine 5'-phosphoric acid (deoxythymidylic acid; dTMP)

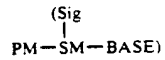
The special nucleotides in accordance with this invention, as indicated hereinabove, in addition to the P, S and B moieties (PM, SM and BASE, respectively), include a chemical moiety Sig coavalently attached to the P, S and/or B moieties (PM, SM and BASE, respectively). Of special interest in accordance with the practices of this invention would be those nucleotides having the general formula,

P—S—B—Sig

(PM—SM—BASE—Sig) wherein P(PM) is the phosphoric acid moiety including mono-, di-, tri-, or tetraphosphate, S(SM) the sugar or monosaccharide moiety, B the base moiety (BASE), either a purine or a pyrimidine. The phosphoric acid moiety P(PM) is attached at the 3' and/or the 5' position of the S moiety (SM) when the nucleotide is a deoxyribonucleotide and at the 2', 3' and/or 5' positio when the nucleotide is a ribonucleotide. The base B moiety (BASE) is attached from the N1 position or the N9 position to the 1' position of the S moiety (SM) when the base moiety is a pyrimidine or a purine, respectively. The Sig moiety is covalently attached to the B moiety (BASE) of the nucleotide and when so attached is capable of signalling itself or makes itself self-detecting or its presence known and desirably or preferably permits the incorporation of the resulting nucleotide P - S - B - Sig (PM-SM-BASE-Sig) into or to form a double-stranded helical DNA or RNA or DNA-RNA hybrid and/or to be detectable thereon.

Another special nucleotide in accordance with this invention is characterized by the general formula:





Such nucleotides in accordance with this invention would be characterized as ribonucleotides. The phosphoric acid moiety (PM) is attached at the 2', 3' and/or 5'position of the sugar S moiety (SM) and the base B (BASE) being attached from the N1 position or the N9 position to the 1' position of the sugar S moiety (SM) when said base (BASE) is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the sugar S moiety (SM) and said Sic chemical moiety when attached to said S moiety (SM) is capable of signalling itself or making itself self-detecting or its presence known and preferably permits the incorporation of the ribonucleotide into its corresponding double- stranded RNA or a DNA-RNA hybrid.

Sig component or chemical moiety of the special nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties (PM, SM or BASE, respectively) or attached thereto via a chemical linkage or linkage arm as described in U.S. Pat. No. 4,711,955, as indicated by the dotted line connecting B and A of the nucleotides of U.S. Pat. No. 4,711,955. The various linker arms or linkages identified in U S. Pat. No. 4,711,455 are applicable to and useful in the preparation of the special nucleotides of this invention.

A particularly important and useful aspect of the special nucleotides of this invention is the use of such nucleotides in the preparation of DNA or RNA probes. Such probes would contain a nucleotide sequence substantially matching the DNA or RNA sequence of genetic material to be located and/or identified. The probe would contain one or more of the special nucleotides of this invention. A probe having a desired nucleotide sequence, such as a single- stranded polynucleotide, either DNA or RNA probe, would then be brought into contact with DNA or RNA genetic material to be identified. Upon the localization of the probe and the formation of a double-stranded polynucleotide containing the probe and the matching DNA or RNA material to be identified, the resulting formed double-stranded DNA or RNA-containing material would then be observable and identified. A probe in accordance with this invention may contain substantially any number of nucleotide units, from about 5 nucleotides up to about 500 or more, as may be required. It would appear that 12 matching, preferably consecutive, nucleotide units would be sufficient to effect an identification of most of the DNA or RNA material to be investigated or identified, if the 12 nucleotide sequence of the probe matches a corresponding cooperative sequence in the DNA or RNA material being investigated or to be identified. As indicated, such probes may contain one or more of the special Sig-containing nucleotides in accordance with this invention, preferably at least about one special nucleotide per 5-10 of the nucleotides in the probe.

As indicated hereinabove, various techniques may be employed in the practices of this invention for the incorporation of the special nucleotides of this invention into DNA and related structures. One particularly useful technique referred to hereinabove involves the utilization of terminal transferase for the addition of biotinated dUMP onto the 3' ends of a polypyrimidine or to single-stranded DNA. The resulting product, such as a single-stranded or cloned DNA, which has biotinated dUMP attached to the 3' ends thereof, can be recovered by means of a Sepharose-avidin column wherein the avidin would complex with the biotinated dUMP at the ends of the DNA and be subsequently recovered. In accordance with the practices of this invention hybridization to mRNA could be accomplished in solution and the resulting hybrid recovered via a Sepharose-avidin column and the mRNA recovered therefrom. Similar techniques could be employed to isolate DNA-RNA hybrids. This technique employing terminal transferase for the addition of the special nucleotides in accordance with this invention is widely applicable and the resulting modified nucleotides containing the special nucleotides in accordance with this invention including the special biotinated nucleotides or the special glycosylated nucleotides could be selectively recovered via complexing with avidin upon a Sepharose-avidin column or complexing with a lectin, such as Concanavalin A or a Sepharose-Concanavalin A column.

Illustrative of the practices of this invention, biotinated dUTP was added to the 3' ends of $d[PT]_4$ as well as single and double stranded DNA employing terminal transferase and the resulting product was purified through G-50 Sepharose and separated on a Sepharose-avidin affinity column. It was found that 69% of the $d[pT]_4$ molecules were biotinated and recovered on the Sepharose-avidin column. The results of this experiment established that terminal transferase added biotinated dUMP to the 3' ends of a polypyrimidine.

The detection of nucleic acids to which specific molecules have been covalently attached can be effected through the use of many naturally occurring proteins to which small molecules are known to specifically bind. In this procedure the small molecules are bound to the nucleotide using the allyl amine side chain. These nucleotides are.then incorporated into specific nucleic acids using a DNA or RNA polymerase or ligase reaction or a chemical linkage. After annealing this probe with a complementary antiprobe sequence, the presence of the probe is assayed for by the specific binding of the protein to the ligand covalently bound to the probe. Examples of protein-ligand reactions that are appropriate for this type of detector system include:

1. Enzymes and allosteric effector or modulator molecules. An example of this is the enzyme threonine dehydratase which is a heterotropic enzyme in that the effector molecule, L-isoleucine, is different than the substrate, L-threonine, J. Monod, J. Wyman and J. P. Changeux (1965), *J. Mol. Biol.* 12:88-118.

2. Effector molecules involved in regulation. An example of this is the specific binding of 3',5-cyclic adenosine monophosphate to the cyclic AMP receptor protein, I. Pastan and R. Perlman, *Science* 169:339-344 (1969). Another example is the lactose repressor molecule and the inducer molecules isopropylthiogalactoside or thiomethylgalactoside. These two inducer molecules are called gratuitous inducers in that they are not metabolized by the enzymes they induce, W. Gilbert and B. Muller-Hill, *Proc. Natl. Acad. Sci.* (US), 70:3581-3584, (1973).

3. Hormone receptors and other receptors on the surface of the cell to which organic molecules will specifically bind. An example of this is the epinephrine-epinephrine receptor system in which epinephrine is bound in a steriospecific manner with a high affinity to the receptor. With this system, since the receptor protein is insoluble in water, it will be imbedded in a lipid bilayer structure as for instance a liposome. Suitable detector systems would include specific enzymes or fluorescent molecules inside or within the lipid bilayer.

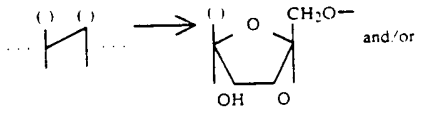
4. Specific ligand binding proteins included in the transport of small molecules. An example of this is the periplasmic binding proteins of bacteria which have been shown to bind many amino acids, glucose, galactose, ribose and other sugars, Pardee, A. *Science.* 162:632-637, (1968); G. L. Hazelbaur, and J. Adler, *Nature New Bio.* 230: 101-104, (1971).

In the above-mentioned examples the ligand bound to the nucleic acid reacts with a naturally occurring protein. The specificity of this reaction resides in the ligand-binding site of the protein.

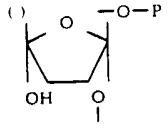
One further example of small molecule interaction with naturally occurring proteins involves the specific binding of coenzyme or other prosthetic molecules to enzymes. Examples of such coenzymes include thiamin

or

where A and B are complementary base pairs, such as a purine, a 7-deazapurine or pyrimidine modified by the addition of an organic moiety Sig in accordance with the disclosures of this invention on the 5 position of the pyrimidine ring or the 7 position of the purine ring or the N^2 of guanine, or the N^6 of adenine or the N^4 of cytosine as described herein. The modifications of the polynucleotides at these positions lead to relatively undisruptive or non-disruptive double-stranded nucleic acid molecules as measured by rates of association and melting points. In the special polynucleotides of this invention employed as inducers of interferon and other cellular or humoral factors or components, such as lymphokines or cytokines, the following groups would be attached thereto as indicated by the formulas,



and/or



In the utilization of the special polynucleotides of this invention, such as the special dsRNA of this invention in the induction process for the production of interferon it has been demonstrated that DEAE-dextran facilitates this operation. It appears that since DEAE-dextran complexes with dsRNA and protects it for nuclease degradation, thereby enhancing interferon induction. It has also been noted that poly rC:rI is taken into cells more efficiently when complexed with DEAE-dextran. Accordingly in the practices of this invention the hydrophobic properties and the ionic or electron charge properties of the special dsRNA of this invention are important factors and capable of manipulation in the applicability of these materials to induce inteferon production. It has been observed that such conditions or factors which promote the induction of interferon also lead to and promote the induction of other cellular or humoral components, such as lymphokines and cytokines. It is apparent, therefore, that the special nucleotides of this invention act as immune modulators and stimulators of the immune response other than simply being effective as inducers of interferon production. Superior agents for the above in accordance with the practices of this invention would include nucleotides wherein the Sig moiety incorporates biotin or streptavidin or avidin.

Poly rI:poly rC complexed poly L-lysine exhibits adjuvant properties and such properties are enhanced and improved in accordance with the practices of this invention when the poly rI and poly rC components are modified to include one or more of the special nucleotides in accordance with this invention.

The preparation of DNA probes in accordance with another aspect of this invention can be carried out in a manner which does not require the preparation or utilization of the special nucleotides described herein. For example, double-stranded DNA can be reacted with a carcinogen or alkylating agent. After the carcinogen has reacted with or alkylated the double-stranded DNA, the resulting modified DNA is melted to produce a DNA hybridizing probe containing the reaction product of the DNA and the carcinogen or alkylating agent. When thus-modified or reacted DNA is employed as a hybridizing probe, any resulting formed double helix or double-stranded DNA would be assayed or searched out by means of a double antibody technique. The primary antibody would be an anti-carcinogen and the secondary antibody would be horseradish-peroxidase conjugated anti-peroxidase antibody. The advantage of this technique is that it would be easy to label the double-stranded DNA. This special approach is indicated hereinabove in the examples accompanying the description of this invention and is generally applicable for the preparation of DNA probes from double-stranded or double helical DNA. However, this procedure is a disruptive procedure involving the modification of the double helical deoxyribonucleotide polymer or DNA.

In the description of the special nucleotides and modified DNA employed or developed in the practices of this invention, mention has been made of mono, oligo and polysaccharides. It is pointed out that derivatives of mono, oligo and polysaccharides are also useful in the preparation of the special nucleotides of this invention. For example, it is possible to modify individual sugar moieties employed in the make-up of the special nucleotides and employ the resulting modified sugar moieties to effect or carry out additional chemical reactions. Such modified mono, oligo and polysaccharide moieties, when employed as the Sig moiety in the preparation of the special nucleotides of this invention, provide an added versatility with respect to the detection of the nucleotides or other compounds containing such modified saccharides either as the sugar S (SM) or as the Sig moiety thereof.

In another aspect of this invention the Sig moiety instead of being attached to a nucleotide could also be attached to proteins. Not only could such proteins be attached to nucleotides or polynucleotides but also such proteins could be identified per se whether attached to a nucleotide or polynucleotide or unattached. In accordance with the practices of this aspect of the invention, a suitable such protein adduct would have the formula,